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2. Patent application number *(The Patent Office will fill in this part)*	0324378.9	17 OCT 2003
3. Full name, address and postcode of the or of each applicant *(underline all surnames)*	University of Edinburgh Old College South Bridge Edinburgh EH8 9YL	
Patents ADP number *(if you know it)*	3977428001	
If the applicant is a corporate body, give the country/state of its incorporation	Great Britain	
4. Title of the invention	Improved control of ES cell self-renewal and lineage specification, and medium therefor	
5. Name of your agent *(if you have one)*		
"Address for service" in the United Kingdom to which all correspondence should be sent *(including the postcode)*	~~MATHYS & SQUIRE 100 Gray's Inn Road London WC1X 8AL United Kingdom~~	Schlich & Co PO Box 48832 London WC2H 9WZ
Patents ADP number *(if you know it)*	1081001 /	08963746001

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Signature Mathys & Squire   Date

MATHYS & SQUIRE   17 October 2003

12. Name and daytime telephone number of person to contact in the United Kingdom   George W Schlich - 020 7830 0000

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IMPROVED CONTROL OF ES CELL SELF RENEWAL AND LINEAGE SPECIFICATION, AND MEDIUM THEREFOR

The present invention relates to media, culture conditions and methods of culturing pluripotent stem cells in order to promote stem cell self renewal and to prevent or control differentiation of the stem cells. The invention further provides methods for deriving, isolating and maintaining homogeneous preparations of pluripotent stem cells. The methods and compositions provided are suitable for culturing and isolating pluripotent stem cells such as embryonic stem (ES) cells, especially mammalian, including human, stem cells.

The establishment and maintenance of in vitro pluripotent stem cell cultures in the presence of medium containing serum and Leukaemia Inhibitory Factor (LIF) is well known (Smith et al. (1988) Nature 336: 688-90). Such methods have been used to maintain pluripotent embryonic stem (ES) cells from strains of permissive mice over many passages. Maintenance and self renewal of pluripotent stem cell cultures is further supported where the stem cells are cultured in the presence of feeder cells or extracts thereof, usually mouse fibroblast cells. Under such conditions it is possible to maintain human ES cells in a pluripotent state over many passages in culture.

A continuing problem in this field is that, despite intense efforts, it remains the case that pluripotent cultures of ES cells can be derived and maintained for extended periods only from a few species and even in those species not from all embryos. In some cases, pluripotent cells can be identified but can not then be maintained in culture for sufficient time to enable study of the cells or their genetic manipulation. This is particularly the case for rodent (other than some strains of mice) cells.

A further problem, until recently, was that ES cells that could indeed be maintained in a pluripotent state in culture over many passages could only be so

maintained using medium that contained serum or serum extract, and hence was undefined, or used cell culture conditions that required the presence of other cells, such as the fibroblast feeder cells used to maintain human ES cells. However, where ES cells are intended to be subjected to subsequent controlled differentiation into desired cell types, it is undesirable to utilise an undefined culture medium or to have heterologous cells present.

The serum typically used in culturing pluripotent stem cells is fetal calf (bovine) serum, which is known to contain a complex mixture of cytokines and other signalling molecules. In order to control differentiation pathways it is undesirable to introduce unknown cytokines to the culture medium whose influence on the eventual outcome of differentiation is unquantifiable, and could be potentially deleterious. Further, each serum batch is unique and introduces variation into culture protocols.

As a result, the ES cells obtained by culture in such complex media, and any differentiated progeny thereof, risk being contaminated by components of the media and/or by cells such as feeder cells that are required to maintain the ES cells. These factors mitigate against development of good manufacturing practices for therapeutic and other applications of ES cells and their progeny.

When deriving a differentiated cell population from an ES cell culture, it is desirable to be able to convert a high proportion of the ES cells into progeny of the same type - i.e. to maintain as homogeneous a population of cells as possible. However, in practice, it is observed that, following differentiation, a cell population is obtained that contains a heterogenous mixture of cells. Hence, it is desirable to be able to carry out differentiation of an ES cell population obtaining a purer population of progeny.

In a prior application by the applicants, culturing pluripotent stem cells, such as ES cells, in serum-free media comprising agonists of the gp130 (e.g. LIF) and TGF-β

purposively activated. With coincident gp130 signalling, especially using a cytokine such as LIF, self renewal of pluripotent cells has been obtained.

It is known from an earlier application by the inventors to promote self renewal using LIF and activation of signalling downstream of a receptor of the TGF-β superfamily. In the present invention, Id gene activity in combination with LIF results in self renewal of ES cells. Hence the invention provides additional means of providing that self renewal signal, namely through a combination of

(i) an agent that increases Id protein activity, said agent being other than an activator of a signalling pathway downstream from a receptor of the TGF-β superfamily; and

(ii) an activator of a gp130 downstream signalling pathway,

The agent of (i) is suitably an extrinsic factor that induces Id gene expression and/or induces id protein activity without acting through a receptor of the TGF-beta superfamily. Examples include fibronectin, agonists of the fibronectin receptor, activators of integrin signalling, nanog, and homologes of all of the aforementioned that induce Id gene expression or Id protein activity.

The methods of the invention are suitable for culture of pluripotent stem cells, especially embryonic stem cells.

In examples below, we have induced expression of an Id gene to promote self renewal. In another embodiment of the invention, also described below, the method comprises genetically manipulating a pluripotent cell so that it expresses an Id gene, for example by introducing into a pluripotent cell a vector comprising an Id gene. Precise control can be achieved using an inducible promoter in the vector. This type of genetic modification is acceptable where the cells are used for drug screens, but may not be so where the cells or progeny are to be used therapeutically - in which case use of extrinsic factors to promote self renewal is preferred.

In specific methods described below in more detail, a method of promoting self-renewal of pluripotent cells in culture, comprises (i) expressing an Id gene or inducing expression of an Id gene, and (ii) activating gp130 downstream signalling. The Id gene can conveniently be expressed episomally

A further aspect of the invention provides the use of a combination of:-

(a)an activator of Id gene expression and/or Id protein activity which results in expression of an Id gene; and

(b) an activator of a gp130 downstream signalling pathway, in promoting self-renewal of pluripotent cells in culture.

An alternative aspect of the invention provides the use of a combination of:-

(a) an Id gene product; and

(b) an activator of a gp130 downstream signalling pathway, in promoting self-renewal of pluripotent cells in culture.

In a specific embodiment of the invention, described in more detail below LIF is included in medium in which an ES cell constitutively expressing Id1 is cultured. Self renewal was enhanced, demonstrating the synergy of LIF and the expressed Id gene.

An advantage of the invention is the direct provision in the cells of an Id gene product, in promotion of self renewal. With direct induction of self renewal comes greater control of self renewal, with less side effects attributable for example to activation of pathways several stages away from the agents of the self renewal mechanism.

Reference to Id genes is intended to encompass the genes as so defined in the literature and is also intended to encompass mimics thereof which exhibit the property of Id gene products, namely that of inhibiting the transcriptional activity of bHLH factors such as myoD and mash1. Id gene activity can suitably be

mimicked by preventing or reducing expression or activity of a bHLH gene or preventing or reducing expression or activity of an E protein. This may be achieved using gene knock-out or inhibitory RNA strategies or by eliminating extrinsic inducing factors. An antisense RNA may be used in one RNA targetting method, or an siRNA based approach can be used.

A signal equivalent to increased Id protein activity may be provided by (i) an inhibitor of a bHLH gene, (ii) an inhibitor of myoD, (iii) an inhibitor of mash1, (iv) increased hes gene activity, (v) increased hes protein activity, and (vi) combinations of one or more of all of the above.

In the art, a factor such as BMP is used to activate one or more signalling pathways downstream from a receptor of the TGF-β superfamily. The present invention differs therefrom in that it relies on direct provision of Id gene activity in the cells, e.g. through a vector expressing an Id gene, or it relies on activation of Id gene expression and/or Id protein activity, other than via a receptor of the TGF-β superfamily or directly mimic the effects of such signalling. The invention can be more targetted than the art and enable greater precision in maintaining a self renewing phenotype than hitherto.

Activation of one or more gp130 downstream signalling pathways can be achieved by use of a cytokine acting through gp130, for example a cytokine or other agonist of the LIF receptor.

Cytokines capable of acting through gp130, and thus of activating gp130 signal transduction, include LIF, CNTF, cardiotrophin, oncostatin M and IL-6 plus sIL-6 receptor. Suitable cytokines include mimetics, fusion proteins or chimaeras that can bind to and/or activate signalling though gp130. The role of cytokines acting through gp130 in the presence of serum is well established, but the capacity of those cytokines to sustain undifferentiated cells in the absence of serum is limited.

The present invention therefore provides, in one embodiment, alternative and/or improved culture of ES cells in medium that is free of serum, serum extract, feeder cells and feeder cell extract. When using the LIF and direct Id gene expression and/or Id protein activity-activating medium of a specific embodiment of the present invention extended passaging of ES cells is possible.

Another advantage of the present culture system is that differentiation of ES cells is reduced compared to culture in the presence of serum. This is significant because often the most pluripotent ES cells tend to differentiate considerably in serum, making their manipulation and expansion problematic. The results show that the culture conditions of the present invention enable ES cells to self-renew in the absence of serum.

Embryonic stem cells have been reported from a number of mammalian sources including mouse (Bradley et al (1984) Nature 309: 255-56), American mink (Mol Reprod Dev (1992) Dec;33(4):418-31), pig and sheep (J Reprod Fertil Suppl (1991);43:255-60), hamster (Dev Biol (1988) May;127(1):224-7) and cow (Roux Arch Dev Biol (1992); 201: 134-141). It will be appreciated that the methods and compositions of the present invention are suitable for adaptation to culturing of other mammalian pluripotent cell cultures, including human, primate and rodent, and avian ES cells.

Specifically, with regard to human ES cells, it is known that human ES cells respond to LIF and therefore the medium and methods of the invention, in which a self-renewal stimulus is obtained in response to a combination of LIF and Id proteins, are of application to human ES cells.

Suitable cell densities for the methods of the invention will vary according to the pluripotent stem cells being used and the natures of any desired progeny. Good results have been obtained by culturing embryonic stem cells in monolayer culture, dissociating the embryonic stem cells and subsequently culturing the

embryonic stem cells in monolayer culture on a culture surface at a density of from 0.2 - 2.5 x 10^4 cells per cm^2, more particularly at a density of from 0.5 - 1.5 x 10^4 per cm^2. The cells proliferate as adherent monolayers and are observed to have a doubling time comparable to ES cells grown in serum-containing media together with LIF.

Typical surfaces for culture of ES cells and their progeny according to the invention are culture surfaces recognized in this field as useful for cell culture, and these include surfaces of plastics, metal, composites, though commonly a surface such as a plastic tissue culture plate, widely commercially available, is used. Such plates are often a few centimetres in diameter. For scale up, this type of plate can be used at much larger diameters and many repeats plate units used.

It is further common for the culture surface to comprise a cell adhesion protein, usually coated onto the surface. Receptors or other molecules on the cells bind to the protein or other cell culture substrate and this promotes adhesion to the surface and it is suggested promotes growth. Gelatin coated plates are commonly available and are suitable for the invention, and other proteins may also be used.

In an embodiment of the present invention, including an agent that suppresses differentiation, such as an inhibitor of the FGF receptor or of MEK/Erk signalling in the culture medium for at least part of the culturing period is found to suppress the tendency of ES cells to differentiate. In one embodiment, the ES cells are cultured in defined serum-free media comprising LIF and an FGF receptor inhibitor for a specified period before the FGF receptor inhibitor is removed and replaced by a direct activator of Smad signalling. Suitable FGF receptor inhibitors include the compounds SU5402 and PD173074. Alternatively, a competitive inhibitor of the FGF receptor can be used, suitably a soluble form of the receptor. Suitable MEK/Erk inhibitors include PD98059, U0126 and PD184352.

In an alternative embodiment, it is an option not to remove the FGF receptor or MEK/Erk inhibitor. Hence, the inhibitor is present in the culture medium for an extended period, either in the presence or absence of inducers of Id proteins. ES cells can thus be grown in culture for at least 20 passages in N2B27 medium in the presence of LIF and an FGF inhibitor. If the inhibitor is not removed from the medium, it is preferred that it is a specific inhibitor and has little or no activity on other receptors.

A second aspect of the invention provides a method of culture of ES cells so as to promote ES cell self renewal, comprising maintaining the ES cells in medium containing:-

(1) (a) an activator of an intracellular signalling pathway, other than one acting through a receptor of the TGF-β superfamily, which results in expression of an Id gene, or (b) an Id gene product; and

(2) an activator of a gp130 downstream signalling pathway.

Methods of the invention can be used for stimulating self-renewal of ES cells in medium which is free of serum and free of serum extract, which cells have previously been passaged in the presence of serum or serum extract. Preferably, such methods are also carried out in the absence of feeder cells and/or feeder cell extracts. For example, culture of ES cells can be carried out comprising the steps of:-

- maintaining the ES cells in a pluripotent state in culture, optionally on feeders, in the presence of a cytokine acting though gp130 and serum or an extract of serum;
- passaging the ES cells at least once;
- withdrawing the serum or the serum extract from the medium and withdrawing the feeders (if present), so that the medium is free of feeders, serum and serum extract; and

- subsequently maintaining ES cells in a pluripotent state in the presence of a direct activator or effector of Id gene expression and/or Id protein activity (other than one acting through a TGF-β receptor) and an activator of a gp130 downstream signalling pathway.

At around the time that the serum or extract of serum is withdrawn from the medium, it is an option to add to the medium an agent that suppresses differentiation, for example, an FGF-receptor inhibitor. It is an option for the inhibitor of differentiation to be withdrawn at the same time as or subsequent to maintenance of the cells in the presence of an Id protein.

The present invention also provides a method of obtaining a transfected population of ES cells, comprising:-

- transfecting ES cells with a construct encoding a selectable marker;
- plating the ES cells;
- culturing the ES cells in the presence of a direct activator or effector of Id gene expression and/or Id protein activity; and an activator of gp130 downstream signalling pathways; and
- selecting for cells that express the selectable marker.

The selectable marker may encode antibiotic resistance, a cell surface marker or another selectable marker as described e.g. in EP-A-0695351.

In a further embodiment, the present invention provides a method of culture of ES cells, comprising the steps of transferring an individual ES cell to a culture vessel, such as an individual well on a plate, and culturing the ES cell in the presence of a direct activator or effector of a Smad signalling pathway and an activator of gp130 downstream signalling pathways, so as to obtain a clonal population of ES cells, all of which are progeny of a single ES cell.

Once a stable, homogenous culture of ES cells is obtained, the culture conditions can be altered to direct differentiation of the cells into one or more cell types selected from ectodermal, mesodermal or endodermal cell fates. Addition of, or withdrawal of cytokines and signalling factors, can enable the derivation of specific differentiated cell populations at high efficiency. Differentiation of an ES cell towards a non-neuroectodermial fate may be achieved by maintaining the ES cell in the presence of a cytokine acting through gp130 and a direct activator or effector of a Smad signalling pathway and then withdrawing the cytokine whilst maintaining the direct activator or effector of a Smad signalling pathway and/or adding a further signalling molecule capable of directing differentiation.

For example, exposure to BMP4 in the absence of LIF leads to induction of mesoderm and endoderm cell types. Withdrawal of agonists of the gp130 and TGF-β signalling pathways and/or blockade of both pathways leads to induction of a neurectodermal phenotype. Alternatively, other signalling factors can be added to the culture conditions to direct other differentiation pathways - for example, activin, sonic hedgehog (shh), Wnts and FGFs.

In use, towards the end of ES cell culture, it is desirable to remove the Smad signal at least one passage before differentiation is initiated, in order to ensure that the signal declines and there is no legacy of the signal during subsequent differentiation. In one embodiment, an FGF receptor antagonist is added to the cultures for one to two passages whilst removing the direct activator or effector of Id gene expression and/or Id protein activity.

Further aspects of the invention provide for cell culture media for self-renewal of ES cells. One such medium comprises:-

- basal medium;
- a direct activator or effector of Id gene expression and/or Id protein activity;
- an activator of gp130 downstream signalling pathways;

- an iron-transporter;

wherein the medium is free of serum and serum extract.

Basal medium is medium that supplies essential sources of carbon and/or vitamins and/or minerals for the ES cells. The basal medium is generally free of protein and incapable on its own of supporting self-renewal of ES cells. The iron transporter provides a source of iron or provides ability to take up iron from the culture medium. Suitable iron transporters include transferrin and apotransferrin.

It is preferred that the medium further comprises one or more of insulin or insulin-like growth factor and albumin (preferably recombinant), and is free of feeder cells and feeder cell extract.

A particular medium of the invention comprises LIF, BMP, insulin, albumin and transferrin, with or without additional basal medium.

The invention also provides cell culture media comprising:-

- a direct activator or effector of Id gene expression and/or Id protein activity; and
- a cytokine acting through gp130.

The culture medium is optionally supplemented with an inhibitor of differentiation of ES cells, or, when differentiation is desired, signalling factors that direct differentiation of ES cells toward a specific phenotype.

It is preferred that the medium is free of serum or serum extract. Most preferably, the medium is fully defined.

In a preferred embodiment of the invention the culture medium comprises the gp130 receptor binding cytokine, LIF, at a concentration of between 10U/ml and

1000U/ml, more preferably between 50U/ml and 500U/ml, even more preferably in the region of 100 U/ml.

The invention further provides a method of deriving a pluripotent cell from a blastocyst, comprising:-

(1) obtaining a blastocyst;

(2) culturing the blastocyst in the presence of an activator of gp130 downstream signalling, to obtain an inner cell mass;

(3) dissociating the inner cell mass;

(4) isolating a cell or cells from the dissociated inner cell mass; and

(5) culturing the isolated cell or cells in the presence of an activator of gp130 downstream signalling and an activator of Id gene expression or a product of Id gene expression.

Preferably, the method comprises culturing the blastocyst in LIF, more preferably for a period of from 2 to 4 days.

The isolated cell or cells are preferably cultured in serum free medium. Typically, the cells are replated as clumps. In examples below, we have obtained good results using a combination of LIF and an agonist of the BMP receptor.

The blastocyst is also preferably cultured in serum free medium, optionally in the absence of an agonist of the BMP receptor.

Still further provided in the invention is a vector, comprising an Id gene operatively linked to a promoter.

The promoter is suitably an inducible promoter, providing for control of expression using extinsic factor. It can be an episomal vector, e.g. as described in examples below.

A further culture medium of the invention is one comprising an agent which induces Id protein expression, other than an agent acting through a receptor of the TGF-β superfamily of receptors. Examples include fibronectin, agonists of the fibronectin receptor, activators of integrin signalling, nanog, and homologes of all of the aforementioned that induce Id gene expression or Id protein activity.

The medium may comprise an Id protein, e.g an Id protein linked to a translocation domain, to facilitate translocation of the Id protein across the cell membrane of a pluripotent cell.

"Translocation domain" means a domain or fragment of a protein which effects transport of itself and/or other proteins and substances across a membrane or lipid bilayer and encompasses native domains and fragments, variants and derivatives that retain this binding function. The latter membrane may be that of an endosome where translocation will occur during the process of receptor-mediated endocytosis. Translocation domains can frequently be identified by the property of being able to form measurable pores in lipid membranes at low pH (Shone *et al.* (1987) Eur J. Biochem. 167, 175-180 describes a suitable test). The latter property of translocation domains may thus be used to identify other protein domains which could function as the translocation domain within the construct of the invention. Examples of translocation domains derived from bacterial neurotoxins are as follows:-

Botulinum type A neurotoxin	– amino acid residues (449 - 871)
Botulinum type B neurotoxin	– amino acid residues (441 - 858)
Botulinum type C neurotoxin	– amino acid residues (442 - 866)
Botulinum type D neurotoxin	– amino acid residues (446 - 862)
Botulinum type E neurotoxin	– amino acid residues (423 - 845)

Botulinum type F neurotoxin – amino acid residues (440 - 864)
Botulinum type G neurotoxin – amino acid residues (442 - 863)
Tetanus neurotoxin – amino acid residues (458 - 879)

"Translocation" in relation to translocation domain, means the internalization events which occur after binding to the cell surface. These events lead to the transport of substances into the cytosol of cells.

A composition for delivery of a factor to an ES cell therefore comprises:-

the factor, and

a translocation domain that translocates the factor into the ES cell, suitably a H_N domain of a clostridial toxin.

The translocation domain can also be selected from (1) a H_N domain of a diphtheria toxin, (2) a fragment or derivative of (1) that substantially retains the translocating activity of the H_N domain of a diphtheria toxin, (3) a fusogenic peptide, (4) a membrane disrupting peptide, and (5) translocating fragments and derivatives of (3) and (4).

Yet further provided in the invention is use of an agent that increases Id protein activity in a pluripotent cell, in promoting self-renewal of the pluripotent cell.

The agent is suitably as described elsewhere herein, and may be one that increases the amount of Id protein in the cell or enhances the activity of Id protein in the cell.

It will be appreciated by a person of skill in the art that activation of signalling pathways downstream from a receptor of the TGF-β superfamily can be effected by either upstream agonists of the TGF-β receptor (e.g. receptor ligands), constitutively active receptors, or activated downstream components of the signalling pathway, for example the SMAD signal transduction molecules.

Likewise upstream effectors (eg. cytokines) and downstream effectors (eg. Stats) of the gp130 signal transduction pathway are capable of activating this pathway also. Thus, embodiments of the invention which refer to activation of signalling downstream of a TGF-β receptor, for example the methods of ES cell derivation, embrace all compositions comprising molecules capable of activating TGF-β receptor superfamily signalling pathways, preferably acting through the BMP receptor, in order to promote self renewal of pluripotent stem cells. Suitable ligands for the BMP receptor include BMPs and GDF.

It is further preferred, according to the invention, that culture of cells is carried out in an adherent culture, and in examples of the invention it has been found that following maintenance of cells in a pluripotent state, differentiation can be induced with a high degree of uniformity and with high cell viability. Adherent cultures may be promoted by the inclusion of a cell adhesion protein, and in specific examples of the invention gelatin has been used as a coating for the culture substrate.

It is also preferred to culture pluripotent cells according to the invention in monolayer culture, though it is optional for cells to be grown in suspension culture or as pre-cell aggregates; cells can also be grown on beads or on other suitable scaffolds such as membranes or other 3-dimensional structures.

A further component of medium for culture of pluripotent cells according to the invention, and which is preferred to be present, is a factor promoting survival and/or metabolism of the cells. In a specific embodiment of the invention, cells are cultured in the presence of insulin. An alternative factor is insulin-like growth factor and other such survival and/or metabolism promoting factors may alternatively be used.

Culture medium used in the examples of the invention preferably also comprises serum albumin. This can be used in purified or recombinant form, and if in a recombinant form this has the advantage of absence of potential contaminating

factors, cytokines etc. The culture medium does not need to contain serum albumin and this component can be omitted or replaced by another bulk protein or by a synthetic polymer (polyvinyl alcohol) as described by Wiles et al.

A particularly preferred medium of the invention is one that is fully defined. This medium does not contain any components which are undefined, that is to say components whose content is unknown or which may contain undefined or varying factors that are unspecified. An advantage of using a fully defined medium is that efficient and consistent protocols for culture and subsequent manipulation of pluripotent cells can be derived. Further, it is found that maintenance of cells in a pluripotent state is achievable with higher efficiency and greater predictability and that when differentiation is induced in cells cultured using a defined medium the response to the differentiation signal is more homogenous then when undefined medium is used.

A medium according to the present invention may be used for culture of pluripotent stem cells from any adult tissue.

Methods of the invention also include a method of obtaining a differentiated cell comprising culturing a pluripotent cell as described and allowing or causing the cell to differentiate, wherein the cell contains a selectable marker which is capable of differential expression in the desired differentiated cell compared with other cell-types, including pluripotent stem cells, whereby differential expression of the selectable marker results in preferential isolation and/or survival and/or division of the desired differentiated cells.

The differentiated cell can be a tissue stem or progenitor cell, and may be a terminally differentiated cell.

The present invention also provides a method of isolating a pluripotent stem cell or an EG or EC cell comprising culturing tissue from an embryo, fetus or adult in medium containing:-

- a cytokine acting through gp130; and
- a direct activator or effector of Id gene expression and/or Id protein activity; and/or
- an inhibitor of a FGF receptor or of MEK/Erk.

Preferably, the medium is a fully defined medium.

Id genes are prominent targets of BMP/Smad signalling in undifferentiated ES cells. Ids are negative helix loop helix factors that sequester E proteins to prevent the transcriptional activity of bHLH factors such as myoD and mash1 (Jen et al., 1992; Lyden et al., 1999). They can also interact with and inhibit Pax and Ets transcription factors (Norton, 2000). In a specific embodiment of the invention, ES cells transfected with *Ids* self-renew in serum-free culture on addition of LIF alone, establishing that a critical contribution of BMP/Smad is to induce *Id* expression.

On LIF withdrawal, Id expressing ES cells readily differentiate but do not give rise to neural precursors. Thus Id proteins act in a lineage-specific manner, suppressing neural determination with little or no effect on mesoderm or primitive endoderm commitment. Ids therefore contribute to self-renewal by complementing the blockade of other lineages by STAT3 (Figure 7). At least part of Id function may be to block the action of prematurely expressed pro-neural factors. Ids may thus act to insulate the stem cell from functional consequences of lineage priming (Hu et al., 1997).

LIF/STAT3 and BMP/Smad hence act in combination to sustain ES cell self-renewal. These two pathways also mediate ventralisation of the *Xenopus* embryo (Nishinakamura et al., 1999). In that case, each appears to be sufficient

independent of activity of the other, with no evidence of cross-regulation between STAT3 and Smad1.

The homoedomain protein Nanog can bypass the requirement for activation of STAT3 in serum-containing medium (Chambers et al., 2003). Nanog can also be used to replace the requirement for BMP/serum stimulation, at least in part by conferring constitutive expression of Id.

There now follows illustrative examples of the invention, accompanied by drawings in which:-

Fig. 1 shows LIF plus BMP sustain ES cell self-renewal in serum-free medium;

Fig. 2 shows clonogenicity, potency and derivation of ES cells in N2B27 with LIF plus BMP;

Fig. 3 shows BMP signalling in ES cells;

Fig. 4 shows expression and function of Ids in ES cells;

Fig. 5 shows Id suppresses neural differentiation and is required for ES cell self-renewal;

Fig. 6 shows Nanog bypasses requirement for BMP/serum to induce Id; and

Fig. 7 shows cooperative lineage restriction by BMP/Id and LIF/STAT3

In more detail, and referring to the examples set out below, Figure 1 shows LIF plus BMP sustain ES cell self-renewal in serum-free medium:-

A. Phase contrast and fluorescent images of *Oct4*-GiP cells cultured in N2B27 with the indicated factors. TuJ1 immunostaining detects neuronal differentiation, green fluorescence reflects activity of the Oct4 promoter in undifferentiated ES cells. Bar: 50μm

B. Plot of cumulative *Oct4*-GFP positive undifferentiated ES cell numbers during progressive passaging in conventional medium with FCS plus LIF or in N2B27 with LIF (10ng/ml) plus BMP4 (10/ng/ml). Cultures were passaged every 48 hours using cell dissociation buffer and replated at 4×10^5 cells per $10 cm^2$ well.

The number of GFP positive cells was determined by FACS analysis at each passage.

C. RT-PCR analyis of Oct4, Nanog, T (brachyury), and Sox1 mRNAs in (1) ES cells in N2B27 with LIF plus BMP for 6 passages, (2) ES cells cultured in serum with LIF, (3) day 8 embryoid bodies (4) day 8 embryoid bodies with retinoic acid treatment.

Figure 2 shows clonogenicity, potency and derivation of ES cells in N2B27 with LIF plus BMP:-

A. CAG-*taugfp* transfectant colony isolated by electroporation of E14Tg2a cells and selection in puromycin.

B. Single CAG-*taugfp* transfectant ES cell and derivative colony.

C. Mid-gestation foetal chimaera produced from TP6.3 ES cells after 6 passages in N2B27 with LIF plus BMP. GFP fluorescence marks ES cell progeny.

D. Male chimaera from CAG-*taugfp* transfected ES cell with C57Bl/6 mate and offspring. Agouti coat colour denotes ES cell origin of offspring.

E. Colony of first passage SF1 ES cells derived in N2B27 with LIF plus BMP.

F. Chimaeras generated from SF1 ES cells

Bar: 50µm

Figure 3 shows BMP signalling in ES cells:-

A. Reverse transcription-PCR analysis of RNA samples from Oct-GiP cells (1) in N2B27 with LIF plus BMP, passage 6, (2) in serum plus LIF, no reverse transcriptase control (3) in serum plus LIF, (4) day 1 after plating in N2B27 without LIF or BMP, (5) day 5 without LIF or BMP.

B. Immunoblots showing Smad1, erk and p38 response to mock treatment (non) or stimulation with LIF, BMP, or LIF plus BMP for 15 minutes or 1 hour after overnight culture in N2B27.

C. Immunoblot showing STAT3 tyrosine phosphorylation response to LIF, BMP, and LIF plus BMP.

D. Smad7 episomal transfectants differentiate and express neural precursor (*Sox1*-GFP) and neuronal (TuJ) markers in the presence of serum and LIF

E. SB203580 (30μM) p38 inhibitor does not suppress either self-renewal in LIF plus BMP or neural differentiation in LIF alone. Oct4-GFP marks undifferentiated ES cells and TuJ1 immunostaining identifies neurons.

F. Co-immunoprecipitation of active Smad1 and STAT3 in ES cells. Left panel: FLAG immunoprecipitates following transfection with FLAG-tagged Smad1. Right panel: STAT3 immunoprecipitates from non-manipulated ES cells. Cells were stimulated as indicated for 1 hour.

Bar: 50μ

Figure 4 shows expression and function of Ids in ES cells:

A. LightCycler reverse transcription PCR analyses of gene induction in response to LIF, BMP, or LIF + BMP. ES cells were cultured overnight in N2B27 alone, then stimulated for 45 minutes.

B. Northern hybridisation of Id mRNA expression in Oct4-GiP cells. Con: steady state ES cells maintained in serum containing medium plus LIF. Lanes 2-11 cells cultured overnight in N2B27 without factors then stimulated as indicated for 45 minutes. Fn, fibronectin.

C. Steady state level of Id1 protein in 46C ES cells transfected with vector alone and cultured in serum-containing medium with LIF, and overexpression in Id1 and fld1 stable integrant clones and after episomal supertransfection of 46C/T cells. The latter blot was exposed for only 10 seconds. Transfected Id1 is FLAG tagged and therefore has retarded migration compared with endogenous Id1.

D. In situ hybridisation of Nanog and Oct4 mRNA in Id1 stable integrant ES cell colonies cultured in N2B27 plus LIF. Equivalent results were obtained with Id2 and Id3 transfectants. Bar: 50μm

Figure 5 shows Id suppresses neural differentiation and is required for ES cell self-renewal:-

A. Phase contrast and GFP fluorescence images of vector and Id3 stable integrant 46C clones after 6 days differentiation in N2B27 without added factors. Id1 and Id2 transfectants showed similar suppression of neural differentiation.

B. Upper panels: fId1 transfectant 46C cells form self-renewing colonies in N2B27 with LIF alone. Middle panels: after Cre excision fId1C cells differentiate in LIF and require LIF plus BMP for ES colony formation. Lower panels: GFP expression in fId1C colonies driven by the constitutive CAG unit after excision of the floxed Id1-STOP cassette.

C. fId1 cells undergo non-neural differentiation on withdrawal of LIF in N2B27 and do not activate *Sox1*-GFP or express TuJ. After Cre excision, fId1C cells show restored differentiation of TuJ positive neuronal cells. (*Sox1*-GFP cannot be specifically detected in fIdC cells due to the constitutive activation of GFP)

D. Reverse transcription PCR analysis of mash1 and ngn2 expression in ES cells and during neural differentiation. Samples as in Figure 3A.

E. Overexpression of E47 blocks ES cell self-renewal, which can be rescued by increased Id1. 46C/T ES cells were supertransfected with E47 or co-supertransfected with E47 plus Id1 episomal expression vectors and cultured for 6 days under dual puromycin and zeocin selection in serum-containing medium with LIF.

F. Increased E47 overcomes Id1 suppression of neural differentiation. 46C/T ES cells were supertransfected as in E then 24 hours after transfection transferred into N2B27 without added factors and cultured for 6 days under dual selection. Bar: 50µm

Figure 6 shows Nanog bypasses requirement for BMP/serum to induce Id:-

A. EF4C cells were cultured for 6 days in N2B27 or in N2B27 plus BMP. EF4 Nanog transfectants were cultured under the indicated conditions for 6 passages and then photographed. Bar: 50µm

B. Northern hybridisation of Id1 and Id3 mRNAs in E14Tg2a parental ES cells and EF4 Nanog transfectants in serum plus LIF (Con) or overnight in N2B27 without factors.

Figure 7 shows cooperative lineage restriction by BMP/Id and LIF/STAT3:- ES cell self-renewal requires suppression of lineage commitment. Id genes induced by BMP or other signals blockade entry into neural lineages, which is otherwise only partially prevented by LIF/STAT3. In parallel the capacity of BMP to induce mesodermal and endodermal differentiation is constrained by STAT3, probably involving direct as well as indirect mechanisms. Withdrawal of LIF therefore results in a switch in BMP action from supporting self-renewal to promoting lineage commitment.

EXAMPLES

Foetal calf serum is important for viability of undifferentiated ES cells in minimal media (Wiles and Johansson, 1999). However, in enriched basal media containing N2 and B27 supplements ES cell viability remains high (Ying and Smith, 2003). This allowed us to examined whether LIF is capable of driving continuous cycles of self-renewal in the absence of serum factors.

In N2B27 medium alone adherent ES cells efficiently convert into *Sox1* positive neural precursors (Ying et al., 2003). LIF reduces but does not eliminate neural differentiation under these conditions. Upon successive passaging in N2B27 medium plus LIF we found that following an initial increase, the number of undifferentiated ES cells reached a plateau and then began to decline after 2-3 passages. This finding was reproduced with several different ES cell lines. Many cells in these cultures had morphology of neural precursors or immature neurons. Neural differentiation was confirmed by activation of the *Sox1*-GFP neural reporter in 46C ES cells (Ying et al., 2003). These observations indicate that additional signalling pathways to LIF/STAT3 are required to promote ES cell self-renewal and in particular to suppress neural determination.

BMPs are well known anti-neural factors in vertebrate embryos (Wilson and Hemmati-Brivanlou, 1995; Wilson and Edlund, 2001) and have been shown to

antagonise neural differentiation of ES cells (Tropepe et al., 2001; Ying et al., 2003). BMP alone promotes differentiation of ES cells into non-neural fates (Johansson and Wiles, 1995; Wiles and Johansson, 1999; Ying et al., 2003) and therefore initially appears unlikely as a candidate self-renewal factor. However, we examined whether addition of BMP might contribute to an inhibition of differentation in conjunction with co-stimulation by LIF. We found that the combination of LIF plus BMP4 (or BMP2) enhanced self-renewal resulting in highly pure populations of undifferentiated ES cells after 2 or 3 passages in N2B27 (Figure 1A). These cultures could subsequently be expanded for multiple passages with no deterioration in growth rate or viability and no neural differentiation (Figure 1 A, B). This response was observed in each of 11 different ES cell lines, originating from three independent derivations. The representation of Oct4 positive undifferentiated cells and the population doubling time were slightly higher than obtained in serum plus LIF (Figure 1B). ES cell status was confirmed by expression of SSEA-1 and alkaline phosphatase (not shown), and of mRNAs for ES cell specific transcription factors Nanog and Oct4 with absence of markers of mesoderm (T) and neuroectoderm (Sox1) (Figure 1C).

The N2 and B27 components improve viability but are not essential for self-renewal. In basal medium supplemented only with transferrin, self-renewal and undifferentiated ES cell expansion can be sustained for multiple passages by LIF plus BMP, but not by LIF alone. The requirement for BMP is therefore not induced by a component in B27.

We tested the BMP relative growth and differentiation factor-6 (GDF-6) and found that it similarly supported ES cell self-renewal in the presence of LIF (Figure 1A). This is not a general feature of the TGF-β superfamily, however, but is restricted to BMP receptor ligands. TGF-β1 had no discernible effect on ES cells, whilst activin increased viability and/or proliferation but did not suppress differentiation.

To test the efficiency of ES cell propagation supported by LIF plus BMP, we undertook electroporation and selection of stable transfectants. Colonies stably expressing *tauGFP* were readily isolated (Figure 2A) and could be amplified into bulk cultures demonstrating the feasibility of using this serum-free system in genetic manipulation protocols.

Self-renewal of isolated ES cells was then investigated. Single ES cells were transferred to 96-well plates in N2B27 with addition of LIF only or of LIF plus BMP4 (Figure 2B). A single colony that formed in the presence of LIF alone contained a high proportion of differentiated cells and could not be expanded further. In contrast, undifferentiated colonies formed in 12/192 wells in LIF plus BMP4 and 10 of these were amplified without serum (Table).

ES cells cultured in LIF plus BMP maintained a diploid chromosome complement after multiple generations. They also retained differentiation potential. Withdrawal of both LIF and BMP resulted in neural differentiation. Removal of LIF with retention of BMP caused differentiation into sheets of flattened epithelial-like cells. Thus the self-renewal response to BMP remains dependent on continuous LIF signalling.

The definitive functional attribute of mouse ES cells is their capacity to re-enter embryonic development and contribute to the full repertoire of differentiated tissues in chimaeric mice. We injected GFP reporter ES cells into mouse blastocysts after propagation in N2B27 with LIF plus BMP for 3 weeks. Analysis at mid-gestation identified several chimaeras with high ES cell contributions to a range of tissues (Figure 2C). As a more rigorous test we used ES cells transfected with *taugfp* and selected and expanded in LIF plus BMP. Liveborn chimaeras were obtained and two male animals transmitted the ES cell genome (Figure 2D).

Derivation of ES cells without feeders or serum.

We investigated whether the response to BMP may be an adaptation of established ES cells to culture or is manifest during the initial stages of ES cell derivation. We plated blastocysts in N2B27 supplemented with BMP plus LIF. After several days expanded inner cell masses (ICMs) were dissociated and replated in the same culture conditions. In initial trials ES cell colonies were not obtained following ICM dissociation after 5-6 days in culture, the standard timing for ES cell derivation (Nichols et al., 1990; Robertson, 1987). However, in the absence of serum and presence of BMP the ICM exhibits reduced growth and more rapid onset of overt differentiation. Therefore we subsequently dissociated the ICM after only 4 days of blastocyst culture in LIF only and added BMP4 on replating. Under these conditions primary ES cell colonies did form (Figure 2E). These could be passaged and expanded as morphologically undifferentiated ES cells. One line (SF1) was characterised further. Upon withdrawal of LIF and BMP, SF1 ES cells underwent neural differentiation in vitro. Moreover, SF1 cells produced extensively chimaeric mice (Figure 2F). Twelve chimaeras were all male, indicative of sex conversion by highly contributing XY ES cells (Bradley et al., 1984).

Hence, we derived ES cells in accordance with the invention by culturing replated cells in the presence of gp130 signalling and an activator of downstream signalling from a receptor of the TGF-β superfamily.

Undifferentiated ES cells express functional BMP signalling machinery.

Single cell cloning and the near-complete absence of differentiation in LIF plus BMP cultures suggested to us that the effect of BMP is likely to be directly on ES cells rather than mediated via differentiated progeny. However, previous studies reporting BMP receptor expression and BMP responsiveness during ES cell differentiation (Adelman et al., 2002; Hollnagel et al., 1999) have not established whether ES cells in the undifferentiated state can actually respond to BMP. To

confirm this we used selection for activity of an *Oct4* transgene (Ying et al., 2002) to purify undifferentiated cells for RNA and protein analyses.

BMPs act through heterodimers of type 1 and type II serine/threonine kinase receptors (Shi and Massague, 2003). Undifferentiated ES cells show little or no type I *Bmprlb* mRNA, but express both type I *Bmprla* and type II *Bmprll* receptor mRNAs (Figure 3A). BMP4 and GDF6 transcripts are also readily detectable in undifferentiated ES cells. The principal effectors downstream of the BMP receptors are the Smad transcription factors (Attisano and Wrana, 2002; von Bubnoff and Cho, 2001). R-Smads 1, 5 and 8 are recruited to and phosphorylated by the active BMP receptor complex and then combine with Smad4 and translocate to the nucleus. We investigated Smad activation by immunoblotting using antibody specific for the active serine phosphorylated form of Smad1. Increased phosphorylation of Smad1 in undifferentiated ES cells is apparent after BMP4 addition (Figure 3B). BMP stimulation also enhances the basal activation of p38 and, by one hour, of erk mitogen-activated protein kinases (Figure 3B).

These data establish that undifferentiated ES cells possess the signal transduction machinery for responsiveness to BMP stimulation and furthermore that they may have the potential for autocrine stimulation via BMP4 and GDF production.

BMP supports self-renewal through Smad activation.

The self-renewal action of LIF is mediated via the transcription factor STAT3 (Matsuda et al., 1999; Niwa et al., 1998). BMP alone does not activate STAT3 measured by phosphorylation of tyrosine 705 (Figure 3C). Nor does it increase STAT3 activation by LIF. Erk activation downstream of gp130 is not required for ES cell self-renewal but appears to be a pro-differentiative signal (Burdon et al., 1999a). Thus reduced erk activity facilitates ES cell derivation (Buehr and Smith, 2003) and promotes self-renewal (Burdon et al., 1999b). Erk activation in response to LIF was not appreciably inhibited by the presence of BMP, however

(Figure 3B). These data indicate that BMP does not modulate gp130 signal transduction in ES cells, implying that a BMP signalling pathway contributes directly to self-renewal.

We introduced the inhibitory Smad family members, Smad6 and Smad7 (Shi and Massague, 2003; von Bubnoff and Cho, 2001), into ES cells to antagonise BMP signalling. Cells were transfected and grown up under puromycin selection in the presence of serum and LIF. Smad6 or Smad7 expression vectors yielded fewer and smaller ES cell colonies relative to transfections with empty vector. Furthermore Smad6 and even more so Smad7 transfectants expanded poorly after passaging. A high level of differentiation was evident in the transfected cell populations. Neural differentiation is normally suppressed by serum in adherent cultures, but was readily apparent after Smad7 transfection (Figure 3D).

In addition to blocking Smad activity, Smad6/7 can also inhibit the TAK/p38 pathway downstream of BMPR (Kimura et al., 2000). To assess the potential contribution of p38 in ES cells we used the specific inhibitor SB203580 (Cuenda et al., 1995). This reagent had no noticeable effect on the capacity of BMP to support self-renewal (Figure 3E). In LIF only, SB203580 did not alter the balance between self-renewal and neural differentiation, but appeared to enhance overall cell viability, suggesting that in ES cells as in other cell types p38 is pro-apoptotic (Kimura et al., 2000). The Smad pathway is therefore the likely transducer of the self-renewal signal.

A mechanism of cooperative transcriptional regulation between Smad and STAT3 has been characterised in neuroepithelial cells (Nakashima et al., 1999; Sun et al., 2001). This involves formation of a ternary complex bridged by the ubiquitous transcriptional co-activator p300 and results in synergistic activation of glial-specific promoters. We investigated whether a complex containing STAT3 and Smads may be formed in ES cells stimulated with LIF plus BMP. Immunoprecipitation following transfection with FLAG-tagged Smad1 indicated that

activated STAT3 and Smad1 may co-localise (Figure 3F). This conclusion was corroborated by co-immunoprecipitation of endogenous phosphorylated Smad1 and STAT3 following LIF plus BMP stimulation (Figure 3F).

BMP target genes in ES cells.

To effect ES cell self-renewal, BMP/Smad and LIF/STAT3 signalling could operate in parallel on distinct target genes and/or may converge on common target genes, for example via the ternary complex with p300. We used real time RT-PCR to survey candidate genes for induction by LIF, BMP, or LIF plus BMP in Oct-selected ES cells (Figure 4A). Two known LIF targets *tis11* and *c-fos* showed no response to BMP. Two others, *junB* and in particular *socs3*, appeared to be more highly induced by LIF in the presence of BMP. These data suggest that a subset of STAT3 target genes may be responsive to co-stimulation with BMP. However, neither JunB nor Socs3 are candidates for effectors of self-renewal: *junB* null ES cells show no defects (Schorpp-Kistner et al., 1999), and SOCS3 functions as a negative feedback regulator of gp130 signalling (Schmitz et al., 2000) that blocks self-renewal when overexpressed.

We also examined expression of *Id* genes, which encode negative bHLH factors and have been shown to be induced by BMP/Smad in neuroepithelial cells (Nakashima et al., 2001) and C2C12 myoblasts (Lopez-Rovira et al., 2002). *Id* mRNA induction by BMP has also been reported in differentiating ES cell cultures (Hollnagel et al., 1999). We found that *Id1* and *Id3* are strongly induced by BMP (and GDF, data not shown), but not by LIF (Figure 4A). Northern hybridisation confirmed these findings and extended them to Id2 (Figure 4B). Neither activin (data not shown) nor TGF-β1 induce *Id* gene expression indicating that this response is specific to Smads downstream of the BMP receptor.

The *Id* genes are also induced by foetal calf serum and by fibronectin, although to a lesser extent than by BMP (Figure 4B). ES cells cultured in serum show

readily detectable steady state amounts of Id mRNAs. We examined whether fibronectin, which induces Id2 and Id3, could replace BMP in N2B27 cultures. Soluble fibronectin in combination with LIF could expand undifferentiated Oct4-GiP cells for at least 10 passages, although with more differentiation and slower population expansion than in BMP.

Constitutive Id bypasses BMP or serum requirements for ES cell self-renewal

We hypothesized that Id induction may provide a specific restriction of neural differentiation to complement the self-renewal activity of STAT3. Accordingly we prepared expression constructs for Id1, Id2 and Id3 and introduced these into ES cells. Colonies were readily recovered by both episomal supertransfection and conventional stable integration. For Id1, elevated protein expression was confirmed by immunoblotting (Figure 4C). Overexpression of the transgene appears to be associated with a reduction in endogenous Id1 protein, implying operation of a feedback or autoregulatory loop.

Forced Id expression did not impair ES cell self-renewal nor block differentiation in the presence of serum. Under these conditions the transfectants were not overtly different from parental ES cells or empty vector transfectants. In contrast, in serum-free N2B27, Id transfectants whilst remaining LIF-dependent, were liberated from requirement for BMP. These cells proliferated in LIF alone as rapidly and with as little differentiation as parental ES cells in LIF plus BMP. The cultures could be passaged multiple times with no change in undifferentiated morphology or factor dependence. The ES cell phenotype was confirmed by expression of *Oct4* and *Nanog* mRNAs (Figure 4D). As a rigorous test of the capacity of *Id* expression to substitute for serum or BMP/GDF, we plated single cells in N2B27. Undifferentiated passageable colonies formed in LIF alone with comparable frequency (10%) to colony formation from isolated cells in LIF plus BMP (Table).

Id proteins exert a lineage-specific block on ES cell differentiation.

In our cultures, LIF is essential for self-renewal of Id transfectants because Ids do not impose a complete block on ES cell differentiation. If LIF is withdrawn in serum-containing medium, Id transfectant cells differentiate as parental ES cells. In adherent culture they produced mostly flattened epithelial-like cells with some fibroblasts. On aggregation they formed embryoid bodies with activation of mesodermal (T) and endodermal (Hnf4) marker expression (data not shown) and developed spontaneous contractility indicative of cardiomyocyte differentiation. However, in N2B27 in the absence of LIF, Id transfectants behaved differently from other ES cells. Neural differentiation, assessed by morphology and by activation of *Sox1*-GFP was minimal (Figure 5A). Instead the transfectants differentiated into sheets of flattened epithelioid cells, similar to parental ES cells exposed to BMP alone (cf Figure 1A).

We prepared a revertable expression construct to test whether self-renewal and blockade of neural differentiation are dependent on continuous Id expression. We generated 46C ES cells expressing floxed Id1 (fId1 cells) and subsequently a Cre treated derivative clone (fId1C) in which the *Id1* transgene had been excised. After Cre excision, fId1C cells show absence of FLAG-Id1 and restored levels of endogenous Id1 (Figure 4C). fId1 and fId1C cells were plated at clonal density in N2B27 with LIF or LIF plus BMP. fId1 cells formed stem cell colonies efficiently in LIF alone but this ability was lost in fId1C cells which produced only differentiated cells in LIF without BMP (Figure 5B). In N2B27 alone, fId1 cells underwent non-neural differentiation whereas fId1C cells behaved in identical fashion to parental ES cells, generating a high proportion of TuJ positive neurons (Figure 5C).

These observations indicate that Id expression specifically blocks neural lineage commitment and diverts differentiating ES cells into alternative fates, much as observed for BMP treatment in the absence of LIF (Ying et al., 2003). Id

expressing ES cells are thus wholly dependent on LIF/STAT3 for inhibition of non-neural lineage commitment and maintainance of pluripotency.

The neurogenic bHLH transcription factors are known to be antagonised by Id proteins in the developing CNS (Lyden et al., 1999). In vivo these bHLH factors have not been reported prior to neurulation. However, cultured ES cells show expression of mRNAs expected to be found only in differentiating lineages (Ramalho-Santos et al., 2002). We therefore investigated the potential expression of two bHLH genes, *mash1* and *neurogenin2*, in Oct4 selected ES cells. Whilst neurogenin2 mRNA is not detectable above background levels, mash1 mRNA appears relatively abundant (Figure 5D). We propose therefore that Id expression may be necessary to prevent continuous neural differentiation of ES cells triggered by precocious expression of mash1 and other pro-neural bHLH factors. Such action may also encompass non-bHLH partners such as Pax and Ets factors (Norton, 2000).

Id proteins bind to ubiquitous HLH factors, the E proteins, with high avidity (Norton, 2000). Overexpression of either will sequester and block activity of the other. To assess whether Id proteins may normally be required for ES cell propagation we overexpressed the E47 protein by episomal supertransfection either alone or in co-transfection with Id1 or Id3. E47 singly or in co-transfection with empty vector yielded few, very small and sickly colonies (Figure 5E). In contrast, healthy ES cell colonies were generated from co-transfection of *E47* and *Id* vectors. Co-transfectant colonies appeared indistinguishable in serum-containing medium from cells transfected with *Id* alone or with empty vector. This suggests that increased E47 is not intrinsically toxic but has a specific growth inhibitory action due to sequestration of Id. A certain level of free Id may by required for ES cell propagation as observed in other cell types (Norton, 2000). When transferred to N2B27 without LIF or BMP, the co-transfectants underwent neural rather than non-neural differentiation, shown by activation of *Sox1*-GFP (Figure 5F). Thus E47 neutralises the neural suppression effect of Id. This is

consistent with the suggestion that Id acts to limit availability of E proteins for partnering with proneural bHLH factors.

Nanog can bypass requirements for BMP or serum.

Increased levels of the variant homeodomain protein Nanog render ES self-renewal independent of LIF/STAT3 in the presence of serum (Chambers et al., 2003). We examined whether LIF and/or BMP are required for self-renewal of Nanog overexpressing ES cells in N2B27. Figure 6A shows that EF4 cells expressing a floxed *Nanog* transgene can be propagated in N2B27 without either LIF or BMP. This behaviour is directly attributable to Nanog, since derivative EF4C cells in which the *Nanog* transgene has been excised by Cre recombinase rapidly undergo neural differentiation. Addition of BMP alone has no apparent effect on EF4 cells, unless cultures are maintained without passage for more than 6 days when some differentiation becomes apparent (see Discussion). On addition of LIF, with or without BMP, EF4 cells adhere more evenly to the culture dish (Figure 6A) and the population doubling rate increases. This accords with previous indications of combinatorial effects of LIF/STAT3 and Nanog in ES cells (Chambers et al., 2003).

Since Nanog renders BMP or serum stimulation redundant, we asked whether EF4 cells express Ids. After overnight culture in N2B27 without LIF or BMP, expression of Id1 and Id3 was markedly down-regulated in parental E14Tg2a cells. By contrast, in EF4 cells Id1 mRNA was reduced though still appreciable, and Id3 mRNA actually increased (Figure 6B). Thus overexpression of *Nanog* can be used to maintain a substantial level of Id expression constitutively.

<u>**Experimental Procedures**</u>

ES cell culture

ES cells were maintained without feeder cells. For serum-free culture, ES cells were plated onto gelatin-coated plates in N2B27 medium (Ying and Smith, 2003) supplemented with 10ng/ml LIF (Sigma) and 10ng/ml BMP4 or 200ng/ml GDF6 (R&D Systems). Cells were passaged every 2-4 days using either enzyme-free Cell Dissociation Buffer (Invitrogen) or 0.025% trypsin/1% chicken serum. Dissociated cells were harvested in N2B27 and pelleted. Supernatant was aspirated and the cell pellet resuspended in N2B27 and replated directly. For single cell cloning, a finely drawn Pasteur pipette pre-loaded with N2B27 was used to pick individual cells into 10μl drops. Drops were then singly transferred to 96-well plates pre-loaded with 150 μl N2B27 per well with LIF or LIF plus BMP4. After 8 days, ES cell colonies were identified and passaged. To produce chimaeras, ES cells were injected into C57Bl/6 blastocysts. Germline transmission was tested by mating male chimaeras with C57Bl/6 females.

Derivation of ES cells in serum free medium

Strain 129 mice were ovariectomised on the third day of pregnancy and embryos in diapause flushed 4 days later (Nichols et al., 1990). Intact blastocysts were plated on gelatin-coated plastic in N2B27 supplemented with LIF (10ng/ml). After 3-6 days the central mass of each explant was picked, rinsed in PBS and placed in a drop of trypsin for a few minutes. The cell mass was picked up in a finely drawn out Pasteur pipette pre-loaded with medium, ensuring minimal carry over of trypsin, and expelled with gentle trituration into a fresh well in N2B27 supplemented with LIF and BMP4 (10ng/ml). Resultant primary ES cell colonies were individually passaged into wells of a 96 well plate. Thereafter, cells were expanded by trypsinisation of the entire culture with centrifugation and aspiration before replating.

RNA analyses

Oct4GiP ES cells (Ying et al., 2002) were cultured in the presence of puromycin for 4-6 days to eliminate differentiated cells. Purified ES cells were cultured in complete medium plus LIF for 24 hours then washed once with PBS and transferred to N2B27 medium overnight prior to stimulation for 45 min. with 20ng/ml LIF, 50ng/ml BMP4, LIF plus BMP4, 10ng/ml TGF-β1 (all R&D Systems) or 15% FCS. Quantitative RT-PCR was carried out using the LightCycler Instrument (Roche). Data were normalised relative to Oct4 amplification. Primer pairs and reaction conditions are available upon request. Northern hybridizations were carried out on 5μg aliquots of total RNA.

Plasmid construction and transfection

Smad6 and Smad7 plasmids were kindly provided by Hitoshi Niwa and FLAG-tagged Id1 by Tetsuya Taga. Mouse Id2, Id3 and E47 open reading frames (ORFs) were amplified by PCR, cloned into pCR2.1, and verified mutation-free by sequence analysis. Expression vectors were introduced into ES cells episomally or by stable integration. Floxed Id1 and Cre-excised derivative ES cell lines were derived using the strategy described by Chambers et al., 2003.

Immunochemistry

Pre-selected Oct4GiP ES cells were transferred to N2B27 medium overnight prior to stimulation with LIF (20ng/ml), BMP4 (50ng/ml) or LIF plus BMP4 for 15min or 1 hour. Phosphorylated stat3, smad1, erk1/2 and p38 were detected by immunoblotting (Cell Signaling Technology). Cell lysis and immunoprecipitation (Nakashima et al., 1997) employed anti-FLAG (Sigma) or anti-Stat3 (Transduction Labs). Immunostaining was performed as described (Ying et al., 2003)

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Table

Propagation of single ES cells in serum-free medium with LIF plus BMP or with LIF alone after Id transfection

	Parental ES Cells		**Id1 Transfectants**	
	LIF	**LIF + BMP4**	**LIF**	**LIF + BMP4**
Number of single cells picked	96	192	192	192

Number of colonies formed at day 8	1	12	19	22
Number of colonies expanded	0	10	16	20

CLAIMS:

1. Use of an Id gene product in promoting self-renewal of pluripotent cells in culture.

2. Use according to Claim 1 of a combination of the Id gene product with an activator of a gp130 downstream signalling pathway.

3. Use of a combination of
   (i) an agent that increases Id protein expression or activity; and
   (ii) an activator of a gp130 downstream signalling pathway,

   in promoting self-renewal of pluripotent cells in culture.

4. Use according to any of Claims 1-3, wherein the activator of a gp130 downstream signalling pathway is LIF.

5. Use according to any of Claims 1-4, wherein the pluripotent cells are embryonic stem cells.

6. Use according to Claim 5 wherein the embryonic stem cells are mouse cells or human cells.

7. Use according to any of Claims 3-6 wherein the agent (i) is selected from fibronectin, agonists of the fibronectin receptor, activators of integrin signalling, nanog, and homologes of all of the aforementioned that induce Id gene expression or Id protein activity.

8. Use according to any of Claims 1-7, comprising inducing expression of an Id gene.

9. Use according to any of Claims 1-8, comprising genetically manipulating a pluripotent cell so that it expresses an Id gene.

10. Use according to any of Claims 1-9, comprising introducing into a pluripotent cell a vector comprising an Id gene.

11. Use according to any of Claims 1-11 wherein the Id gene product is an Id protein.

12. A method of promoting self-renewal of pluripotent cells in culture, comprising (1) expressing an Id gene or inducing expression of an Id gene, and (2) activating GP130 downstream signalling.

13. A method according to Claim 12, comprising expressing an Id gene episomally in the cell.

14. A method according to Claim 13 comprising expressing an Id gene from an episomal vector comprising an inducible promoter.

15. A method according to any of Claims 12-14, comprising stimulating gp130 downstream signalling by culturing the cell in medium comprising a cytokine acting through gp130.

16. A method according to Claim 15 wherein the cytokine is selected from LIF, CNTF, Cardiotrophin, Oncostatin M and a combination of IL-6 plus sIL-6 receptor.

17. Use of a combination of:-

(a) a direct activator or effector of Id gene expression and/or Id protein activity, other than one acting through a receptor of the TGF-β superfamily; and

(b) an activator of a gp130 downstream signalling pathway, in promoting self-renewal of a pluripotent cell in culture.

18. A method of culture of ES cells so as to promote ES cell self renewal, comprising maintaining the ES cells in medium containing:-

(a) a direct activator or effector of Id gene expression and/or Id protein activity, other than one acting through a receptor of the TGF-β superfamily; and

(b) an activator of a gp130 downstream signalling pathway.

19. A method of culture of ES cells, comprising:-

(a) maintaining the ES cells in a pluripotent state in culture, optionally on feeders, in the presence of a cytokine acting though gp130 and serum or an extract of serum;

(b) passaging the ES cells at least once;

(c) withdrawing the serum or the serum extract from the medium and withdrawing the feeders if present, so that the medium is free of feeders, serum and serum extract; and

(d) subsequently maintaining ES cells in a pluripotent state in the presence of:-

(i) a direct activator or effector of Id gene expression and/or Id protein activity, other than one acting through the receptor of the TGF-β superfamily; and

(ii) an activator of a gp130 downstream signalling pathway.

20. A method of obtaining a transfected population of ES cells, comprising:-

(a) transfecting ES cells with a construct encoding a selectable marker operably linked to a promoter that expresses the selectable marker preferentially in ES cell;

(b) plating the ES cells;

(c) culturing the ES cells in the presence of

(i) a direct activator or effector of Id gene expression and/or Id protein activity, other than one activator acting through a receptor of the TGF-β superfamily; and

(ii) an activator of a gp130 downstream signalling pathway; and

(d) selecting for cells that express the selectable marker.

21. A method of culture of ES cells, comprising transferring an individual ES cell to a culture vessel and culturing the ES cell in the presence of

(a) a direct activator or effector of Id gene expression and/or Id protein activity, other than one acting through a receptor of the TGF-β superfamily; and

(b) an activator of a gp130 downstream signalling pathway,

so as to obtain a clonal population of ES cells, all of which are progeny of a single ES cell.

22. A method of directing differentiation of an ES cell towards a non-neurectodermal fate, comprising:-

(a) maintaining the ES cell in the presence of a cytokine acting through gp130 and a direct activator or effector of Id gene expression and/or Id protein activity, other than one acting through a receptor of the TGF-β superfamily; and

(b) withdrawing the cytokine whilst

(c1) maintaining the direct activator or effector of Id gene expression and/or Id protein activity; and/or

(c2) adding a further signalling molecule capable of directing differentiation.

23. A medium for self-renewal of ES cells, comprising:-

(1) basal medium;

(2) a direct activator or effector of Id gene expression and/or Id protein activity, other than one acting through a receptor of the TGF-β superfamily;

(3) an activator of gp130 downstream signalling pathways; and

(4) an iron transporter;

wherein the medium is free of serum or serum extract.

24. A method of deriving a pluripotent cell from a blastocyst, comprising:-

(1) obtaining a blastocyst;

(2) culturing the blastocyst in the presence of an activator of gp130 downstream signalling, to obtain an inner cell mass;

(3) dissociating the inner cell mass;

(4) isolating a cell from the dissociated inner cell mass; and

(5) culturing the isolated cell in the presence of an activator of gp130 downstream signalling and an activator of Id gene expression or a product of Id gene expression.

25. A method according to Claim 24, comprising culturing the blastocyst in LIF.

26. A method according to Claim 24 or 25 comprising culturing the isolated cell in a combination of LIF and an agonist of the BMP receptor.

27. A method according to any of Claims 24-26, comprising culturing the blastocyst for a period of from 2 to 4 days.

28. A method according to any of Claims 24-27 comprising culturing the isolated cell in serum free medium.

29. A method according to any of Claims 24-28 comprising culturing the blastocyst in serum free medium.

30. A method according to any of Claims 24-29 comprising culturing the blastocyst in the absence of an agonist of the BMP receptor.

31. A vector, comprising an Id gene operatively linked to a promoter.

32. A vector according to Claim 31 wherein the promoter is an inducible promoter.

33. A vector according to Claim 31 or 32 which is an episomal vector.

34. A culture medium comprising an agent which induces Id protein expression, other than an agent acting through a receptor of the TGF-β superfamily of receptors.

35. A culture medium comprising an Id protein.

36. A culture medium according to Claim 35, comprising an Id protein linked to a translocation domain, to facilitate translocation of the Id protein across the cell membrane of a pluripotent cell.

37. Use of an agent that increases Id protein activity in a pluripotent cell, in promoting self-renewal of the pluripotent cell.

38. Use according to Claim 37 wherein the agent increases the amount of Id protein in the cell.

39. Use according to Claim 37 wherein the agent enhances the activity of Id protein in the cell.

IMPROVED CONTROL OF ES CELL SELF RENEWAL AND LINEAGE SPECIFICATION, AND MEDIUM THEREFOR

ABSTRACT

Self renewal of pluripotent cells in culture is promoted using a combination of an Id gene product and an activator of a gp130 downstream signalling pathway.

Fig 1

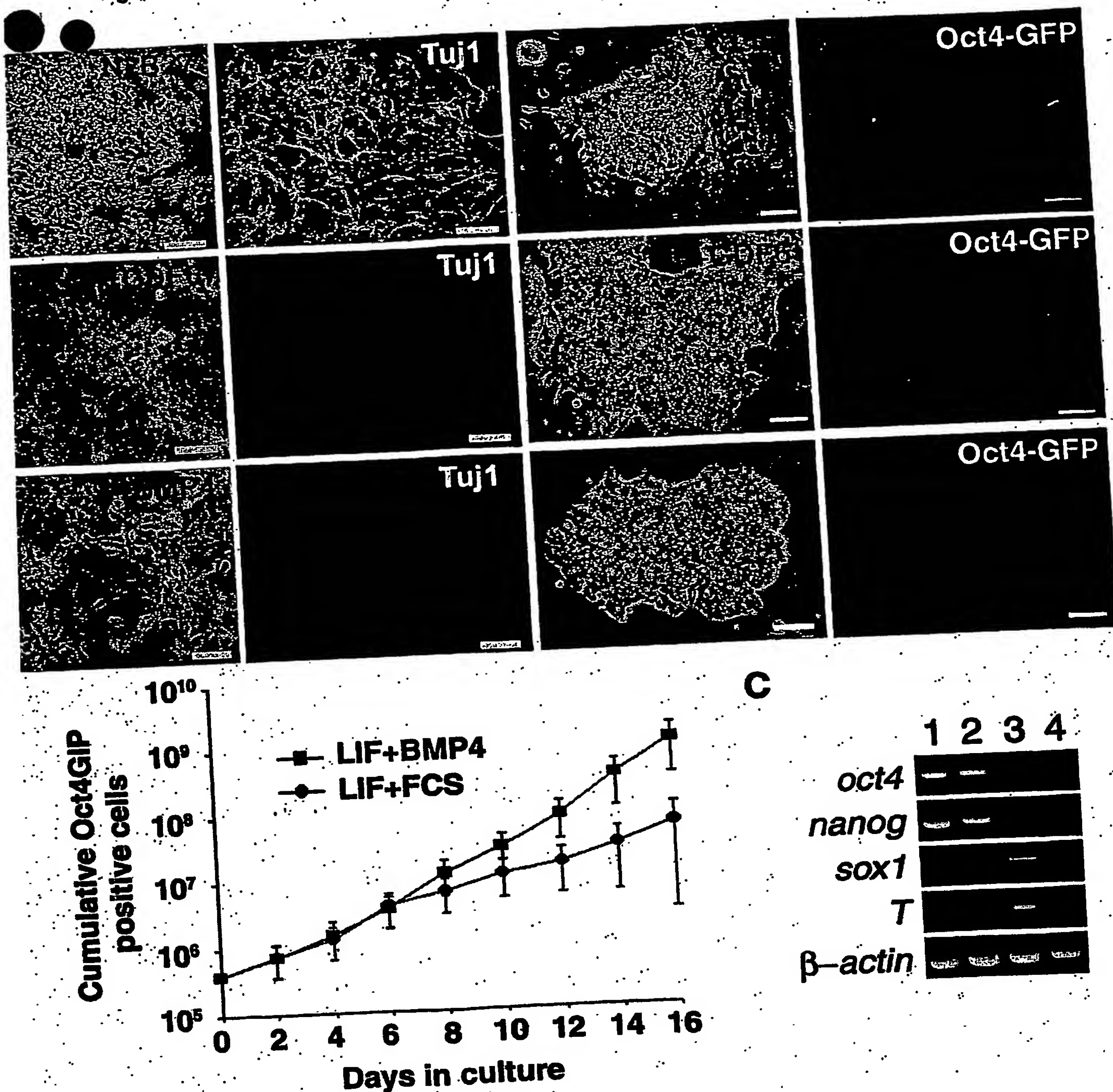

Tuj1
Oct4-GFP
Tuj1
Oct4-GFP
Tuj1
Oct4-GFP
LIF+BMP4
LIF+FCS
Cumulative Oct4GIP positive cells
10^{10}
10^{9}
10^{8}
10^{7}
10^{6}
10^{5}
0 2 4 6 8 10 12 14 16
Days in culture
C
1 2 3 4
oct4
nanog
sox1
T
β–actin

Fig 2

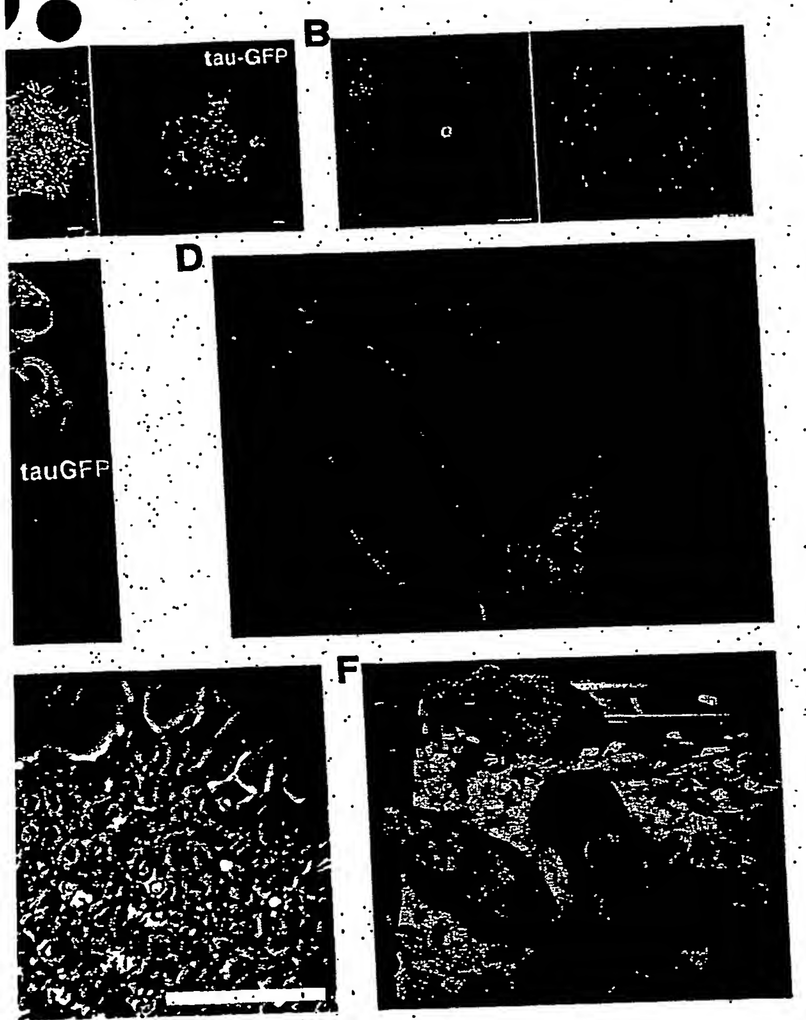
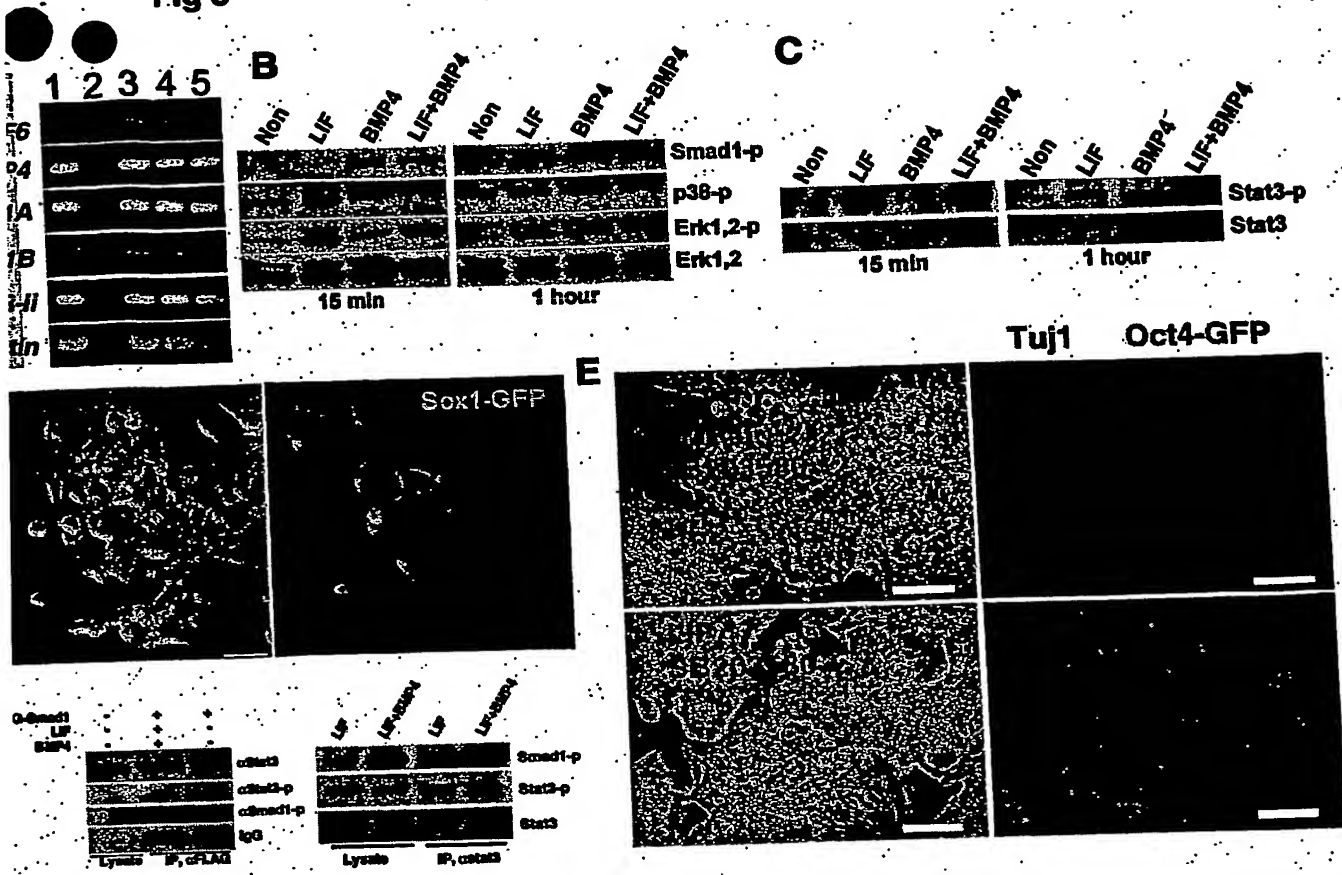
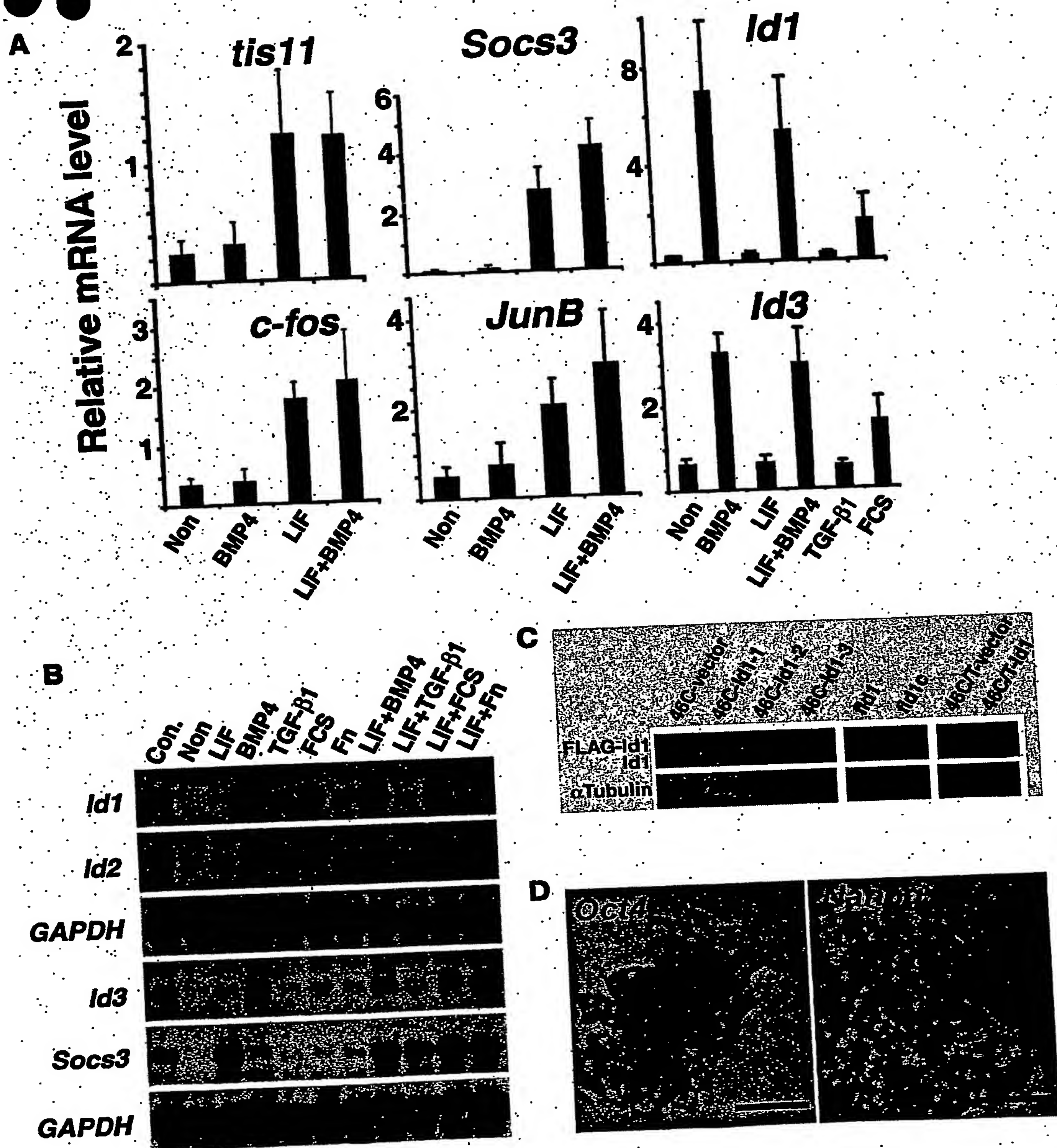


Fig 3



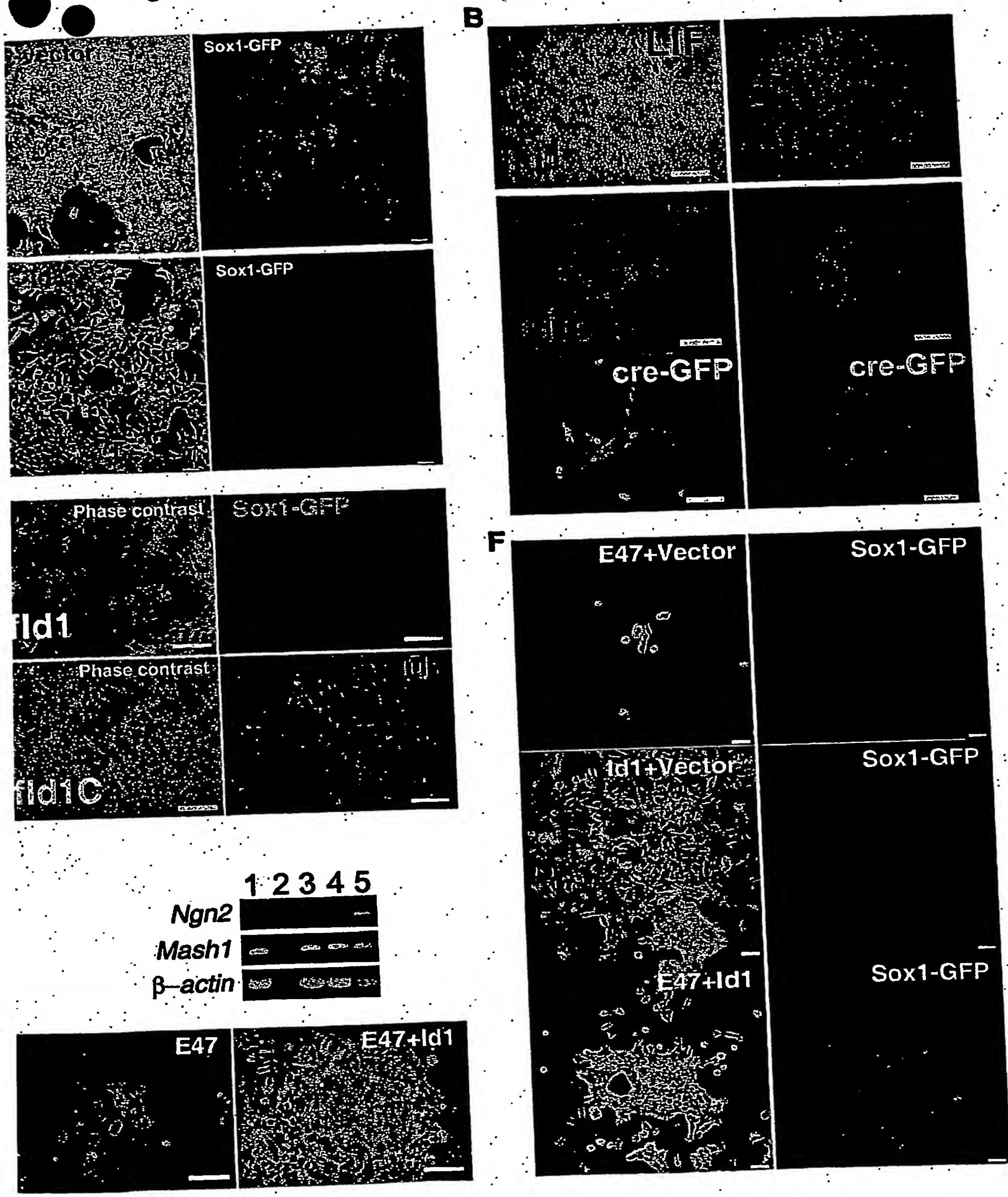
1 2 3 4 5
B
Non
LIF
BMP4
LIF+BMP4
Smad1-p
p38-p
Erk1,2-p
Erk1,2
15 min
1 hour
C
Stat3-p
Stat3
Sox1-GFP
E
Tuj1
Oct4-GFP
Smad1-p
Stat3-p
Stat3
Lysate

Fig 4



A
Relative mRNA level
tis11
Socs3
Id1
c-fos
JunB
Id3
Non
BMP4
LIF
LIF+BMP4
TGF-β1
FCS
B
Con.
Fn
LIF+TGF-β1
LIF+FCS
LIF+Fn
Id2
GAPDH
C
FLAG-Id1
αTubulin
D

Fig 5


Sox1-GFP
Sox1-GFP
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Sox1-GFP
fId1
Phase contrast
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Mash1
β-actin
E47
E47+Id1
B
LIF
cre-GFP
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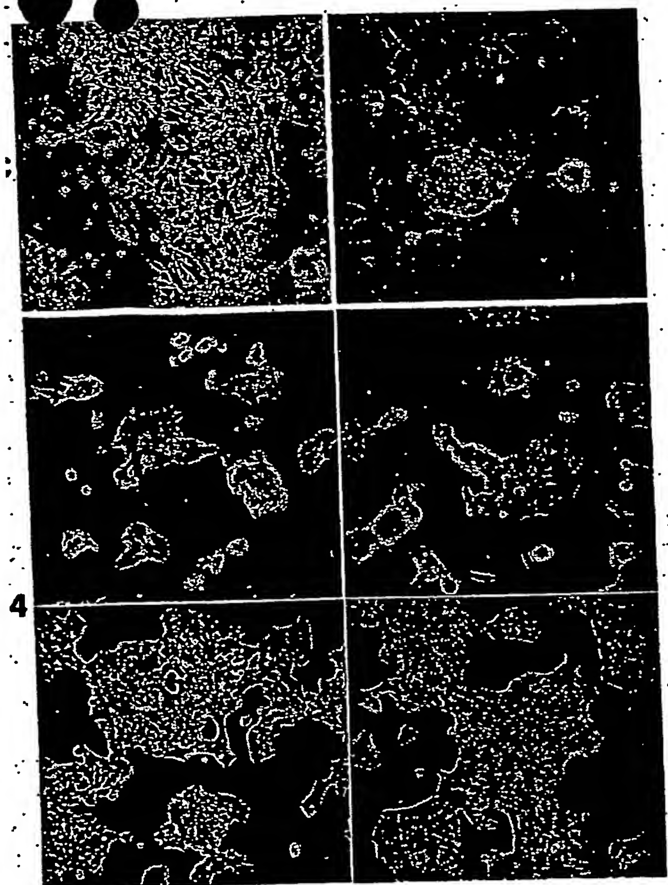
E47+Id1
Sox1-GFP

Fig 6



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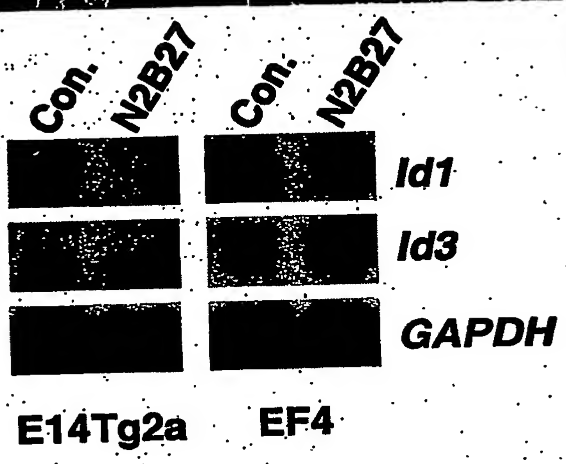
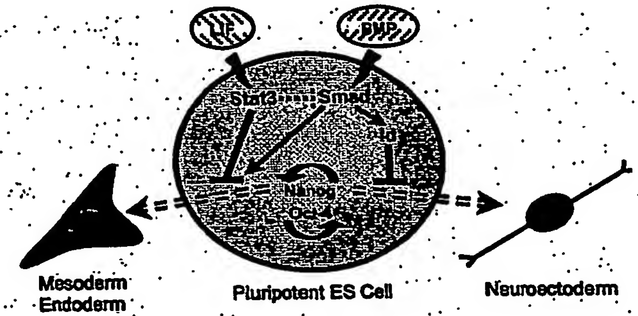


Fig 7


Stat3
Smad
Id
Nanog
Mesoderm
Endoderm
Pluripotent ES Cell
Neuroectoderm